Clear cell sarcomas of the kidney are characterised by BCOR gene abnormalities, including exon 15 internal tandem duplications and BCOR–CCNB3 gene fusion

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Aims: Clear cell sarcoma of the kidney (CCSK) is a rare paediatric renal malignant tumour. The majority of CCSKs have internal tandem duplications (ITDs) of the BCOR gene, whereas a minority have the YWHAE–NUTM2 gene fusion. A third ‘double-negative’ (DN) category comprises CCSKs with neither BCOR ITDs nor YWHAE–NUTM2 fusion. The aim of this study was to characterise 11 histologically diagnosed CCSKs immunohistochemically (with CCND1, BCOR and CCNB3 stains) and genetically.

Methods and results: By next-generation sequencing, 10 cases (90.9%) had BCOR exon 15 ITDs, with positive BCOR immunoreactivity being found in four (36%) or eight (72%) cases, depending on the antibody clone. By reverse transcription polymerase chain reaction, none had the YWHAE–NUTM2 fusion. The DN case had a BCOR–CCNB3 fusion and strong nuclear CCNB3 and BCOR immunoreactivity. Quantitative polymerase chain reaction showed markedly elevated BCOR expression in this case, whereas BCOR ITD cases had lower levels of elevated BCOR expression.

Conclusions: The majority of the CCSKs in our cohort had BCOR ITDs, and none had the YWHAE–NUTM2 fusion. We verified the strong, diffuse cyclin D1 (CCND1) immunoreactivity in CCSKs described in recent reports. BCOR immunoreactivity was not consistently positive in all CCSKs with BCOR ITDs, and therefore cannot be used as a diagnostic immunohistochemical stain to identify BCOR ITD cases. The DN case was a BCOR–CCNB3 fusion sarcoma. BCOR–CCNB3 sarcoma is typically a primary bone sarcoma.
affecting male adolescents, and this is the first report of it presenting in a kidney of a young child as a CCSK. The full spectrum of DN CCSKs awaits more comprehensive characterisation.

Keywords: BCOR internal tandem duplication, CCNB3, clear cell sarcoma of kidney, cyclin D1 (CCND1), gene fusion, immunohistochemistry, paediatric renal tumours

Introduction

Clear cell sarcoma of the kidney (CCSK) is the second most common paediatric renal malignancy, constituting approximately 3% of malignant paediatric renal tumours.1 Recent studies have identified recurrent genetic changes in CCSK.2–11 The largest category comprises CCSKs with internal tandem duplications (ITDs) of the BCOR gene.4,7–9,11 A smaller category (up to 12% of cases) comprises CCSKs with the YWHAE–NUTM2 gene fusion,2,3 which is a recurrent genetic change first described in high-grade endometrial stromal sarcomas (HG-ESSs) of the adult uterus.12,13 The third category consists of CCSKs with neither BCOR ITDs nor YWHAE–NUTM2 fusion;11 this is at present a poorly characterised category that we term ‘double-negative’ (DN) CCSK.

The diagnosis of CCSKs is challenging, because they are morphologically diverse and can mimic other paediatric renal tumours.1,14 One study reported that as many as 50% of CCSKs submitted for central review were initially misdiagnosed as other tumours by the original institutional pathologist.15 Accurate diagnosis is important because the clinical course and treatment of CCSK are different from those of the other paediatric renal tumours. Unlike Wilms tumour, CCSK tends to metastasise to bone, brain, and unusual sites, and is associated with late recurrences.1,16,17 Also, treatment regimens for CCSK incorporate the use of doxorubicin, with its attendant risk of cardiotoxicity, nephrotoxicity, and fertility problems.18

We previously demonstrated the value of strong and diffuse cyclin D1 (CCND1) immunoreactivity as a diagnostic immunohistochemical marker for CCSK,19 and this was corroborated by another study at about the same time.20 In our present study, we extended our investigations to a larger cohort of CCSK cases with the following aims. First, we sought to characterise our cohort of cases in relation to the presence of the YWHAE–NUTM2 fusion and BCOR ITDs. Second, we employed whole transcriptome sequencing (RNA-sequencing) to determine the identity of fusion genes in a case that we had previously identified as having a novel chromosomal translocation by conventional karyotyping,19 and reverse transcription polymerase chain reaction (RT-PCR) to determine whether this translocation was recurrent in any other cases. Third, we examined the value of BCOR immunohistochemistry for the diagnosis of CCSK in the clinical setting, given the findings of a recent study reporting BCOR immunoreactivity in soft tissue sarcomas with BCOR abnormalities (both fusions and ITDs).21 Fourth, we tested for cyclin D1 immunoreactivity in this larger cohort of cases to confirm our previous finding. Finally, having identified a DN CCSK, we sought to investigate this case more extensively in order to determine the underlying genetic changes.

Materials and methods

PATIENT TUMOUR SAMPLES

Four previously reported Singapore CCSK cases19 had sufficient archival tumour material for this study (cases 1–4; Table 1). We included an additional local CCSK case (case 5) and six cases from Shanghai, China (cases 6–11). These cases were clinically characterised in relation to patient gender and age (Table 1), and were reviewed histologically by paediatric pathologists (K.T.E.C. and M.Z.Y.) following histological criteria described in the literature and the 2016 World Health Organisation (WHO) classification of tumours of the urinary system.1,14,22 Formalin-fixed paraffin-embedded (FFPE) material was available for all 11 cases. In addition, snap-frozen material was available for case 2, which is the case with the cytogenetically identified gene translocation. This study was conducted in accordance with local ethical requirements (V06-266, SingHealth Centralised Institutional Review Board approval 2012/450/F).

IMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed on 4-μm-thick full sections. The following antibodies were used: anti-CCND1 (rabbit monoclonal, 1:50 dilution, clone SP4, MA1-39546; Thermo Fisher Scientific, Waltham, MA, USA); anti-BCOR (Abcam) (rabbit polyclonal, 1:50 dilution, ab135801; Abcam, Cambridge, MA, USA); anti-BCOR (Santa Cruz) (mouse...
monoclonal, 1:100 dilution, clone C-10, sc-514576; Santa Cruz, Dallas, TX, USA); and anti-CCNB3 (rabbit polyclonal, 1:400 dilution, HPA000496; Sigma-Aldrich, St Louis, MO, USA). These markers (CCND1, BCOR, and CCNB3) are nuclear stains, and immunoreactivity was assessed on the basis of nuclear staining. To reflect the practicalities and realities of routine diagnostic histopathology practice, only moderate or strong nuclear staining was regarded as positive, and vague or blush-like patterns of reactivity were regarded as negative.

RNA ISOLATION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Unless otherwise stated, total RNA was extracted from scrolls of FFPE tissue blocks with the ReliaPrep FFPE Total RNA Miniprep System (Promega, Madison, WI, USA). Five hundred nanograms of total RNA was reverse transcribed into cDNA with the GoTaq 1-Step RT-PCR kit (Promega) according to the manufacturer’s protocol.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

Fifty nanograms of total RNA isolated from FFPE sections was reverse transcribed into cDNA and used for qPCR based on the GoTaq 1-step RT-qPCR system (Promega). Data S1, Tables S1–S2, Figure S1 details the primer sequences and thermocycling conditions.

TARGETED NEXT-GENERATION SEQUENCING (NGS) OF BCOR IN CCSKS

Genomic DNA from FFPE tissue blocks was purified with the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), and this was followed by quality inspection with an in-house developed multiplex polymerase chain reaction (PCR) assay. Subsequently,

Table 1. Summary of clinical, pathological and genetic findings of the 11 clear cell sarcoma of the kidney (CCSK) cases

<table>
<thead>
<tr>
<th>CCSK case</th>
<th>Gender</th>
<th>Age (years)</th>
<th>CCND1 (IHC)</th>
<th>YWHAE-NUTM2</th>
<th>BCOR ITD</th>
<th>Double-Neg.</th>
<th>BCOR (Abcam)*</th>
<th>BCOR (Santa Cruz)†</th>
<th>BCOR (qPCR)</th>
<th>Other fusions</th>
<th>CCND3 (IHC)</th>
</tr>
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<tr>
<td>1</td>
<td>M</td>
<td>8</td>
<td>Positive</td>
<td>–</td>
<td>Negative</td>
<td>Yes</td>
<td>Negative</td>
<td>Positive</td>
<td>↑↑↑</td>
<td>BCOR-CCNB3</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
<td>No</td>
<td>Negative</td>
<td>Positive</td>
<td>↑</td>
<td>FAM175A-MRP518C GLE1-ODF2 KANSL1-ARL17A PPP1R2-ALKBH5</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>7</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
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<td>Negative</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>4</td>
<td>F</td>
<td>6</td>
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<td>–</td>
<td>Positive</td>
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<td>Negative</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>5</td>
<td>F</td>
<td>4</td>
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<td>–</td>
<td>Positive</td>
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<td>Positive</td>
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<td>ND</td>
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<td>M</td>
<td>2</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
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<td>Negative</td>
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<td>ND</td>
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<tr>
<td>7</td>
<td>M</td>
<td>6</td>
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<td>Positive</td>
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<td>ND</td>
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<tr>
<td>8</td>
<td>M</td>
<td>3</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
<td>No</td>
<td>Positive</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>M</td>
<td>2</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
<td>No</td>
<td>Positive</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
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</tr>
<tr>
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<td>2</td>
<td>Positive</td>
<td>–</td>
<td>Positive</td>
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<td>Negative</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
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<td>Positive</td>
<td>No</td>
<td>Negative</td>
<td>↑</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

Double-Neg., double-negative for YWHAE-NUTM2 fusion and BCOR internal tandem duplication; F, female; IHC, immunohistochemistry; ITD, internal tandem duplication; M, male; ND, not determined; qPCR, quantitative real-time polymerase chain reaction; ↑, low level of transcript up-regulation (<20-fold); ↑↑, moderate level of transcript up-regulation (≥20-fold); ↑↑↑, marked level of transcript up-regulation (≥100-fold).

*Immunohistochemical staining with the BCOR (Abcam, ab135801) rabbit polyclonal antibody.
†Immunohistochemical staining with the BCOR (Santa Cruz, sc-514576) mouse monoclonal antibody.
four pools of GeneRead DNAseq custom-designed pri-

mers (Qiagen, Valencia, CA, USA) were used for tar-

get-enriched amplification based on 60 ng of genomic DNA template. NGS was performed on an Illumina MiSeq sequencer for 500 cycles with the MiSeq reagent kit v2 (Illumina, San Diego, CA, USA). The data generated as FASTQ files were analysed with bioinformatics algorithms (Supplementary protocols; Data S1, Tables S1–S2, Figure S1).^23–28^  

**WHOLE TRANSCRIPTOME SEQUENCING (RNA SEQUENCING)**  
Total RNA was extracted from snap-frozen tumour tissue with the QIAsymphony automated RNA system (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Sample RNA integrity number quality was assessed with the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and ensured to be above 7. The RNA library was prepared by the use of TruSeq Stranded Total RNA with the Ribo-Zero Human/Mouse/Rat kit (Illumina), starting with 500 ng to 1 μg of total RNA. Following cluster amplification of denatured templates, these paired-end templates (2 × 100 bp) were sequenced with the Illumina HiSeq2500 next-generation sequencer (Illumina). See Supplementary protocols (Data S1, Tables S1–S2, Figure S1) for details of the bioinformatics analyses.  

**NANOSTRING-BASED SARCOMA FUSION GENE DETECTION ASSAY**  
We used a laboratory-developed customised CodeSet of gene-specific DNA probes covering 178 fusion variants across 24 sarcoma types, each identified by a unique barcode TagSet. The NanoString Elements fusion assays were performed with 200 ng of total RNA according to the manufacturer’s instructions. This customised CodeSet included probes targeting the YWHAE–NUTM2 and BCOR–CCNB3 fusions. After sample hybridisation, samples were purified and scanned with the nCounter Prep Station and the nCounter Digital Analyser (NanoString Technologies, Seattle, WA, USA), respectively. Data were analysed with NSOLVER (version 3.0). 

**Results**  
**CLINICAL FEATURES**  
The clinical features are summarised in Table 1. The male/female ratio was 3:1. The age range was 2–8 years. All patients were of Asian ethnicity, and comprised seven Chinese, two Malays, and two Indians. 

**CYCLIN D1 IMMUNOHISTOCHEMISTRY**  
Cyclin D1 immunohistochemistry was strongly and diffusely positive in all 11 cases. 

**YWHAE–NUTM2 FUSION STATUS**  
The YWHAE–NUTM2 fusion was not detected in any of the 11 cases by either RT-PCR or the NanoString sarcoma fusion gene detection assay. 

**BCOR EXON 15 ITD STATUS, GENE EXPRESSION, AND IMMUNOHISTOCHEMISTRY**  
Ten of the 11 cases (cases 2–11 in Table 1) (90.9%) had ITDs in exon 15 of BCOR as identified by the amplification of larger PCR products of the ITD region with previously described primers. Sequencing of the PCR products showed ITDs that corresponded to previously described ITDs (Figure 1). There was one DN case (case 1 in Table 1) with neither YWHAE–NUTM2 fusion nor BCOR exon 15 ITD. BCOR NGS did not reveal any other BCOR mutations in all 11 cases. 

BCOR qPCR showed moderately elevated transcript expression in all 10 BCOR ITD cases (cases 2–11) (Figure 2). Case 1 (which was a DN CCSK) had a markedly elevated BCOR expression level. 

BCOR immunohistochemistry with two commercial antibodies did not correspond to BCOR transcript expression levels. With the BCOR (Abcam) antibody, the staining was negative in seven cases, and positive in four cases (36%). With the Santa Cruz BCOR antibody, the staining was negative in three cases and positive in eight cases (72%). Representative images of the histological features and immunohistochemical staining results of both BCOR antibodies and BCOR immunohistochemical staining results of all 11 cases. 

**FUSION GENE DETECTION BY WHOLE TRANSCRIPTOME SEQUENCING**  
Previously, we reported that case 2 harboured a t (3;17)(q29;p11.2) chromosomal translocation. Whole transcriptome sequencing (RNA-sequencing) identified a translocation between PPP1R2 and ALKBH5. In addition, three additional
intrachromosomal gene fusions were identified: FAM175A–MRPS18C (4q21.23), GLE1–ODF2 (9q34.11), and KANSL1–ARL17A (17q21.31). All four gene fusions were validated by Sanger sequencing of RT-PCR-generated amplicons, with primers flanking the fusion breakpoints (Data S1, Tables S1–S2, Figure S1). RT-PCR with the same primers on the other 10 cases showed that none of these gene fusions was recurrent.

**NANOSTRING-BASED SARCOMA FUSION GENE ASSAY AND CCNB3 IMMUNOHISTOCHEMISTRY**

To determine whether any of the CCSKs harbour any other known sarcoma-related gene fusion, we performed the NanoString-based sarcoma fusion assay. This identified a BCOR–CCNB3 fusion in case 1 in which exon 15 of BCOR was fused to exon 5 of CCNB3, corresponding to previously published cases of BCOR–CCNB3 sarcomas. This case had typical histology that corresponded to CCSK (Figure 4A). The BCOR (Santa Cruz) stain was positive (Figure 4B), while the BCOR (Abcam) stain was negative (Data S2). CCNB3 immunohistochemistry was positive (Figure 4C). The BCOR–CCNB3 fusion was confirmed by RT-PCR followed by Sanger sequencing of the amplon product (Figure 4D). CCNB3 immunohistochemical staining on cases 2–5 was negative.

**Discussion**

The first described recurrent genetic alteration in CCSK was the YWHAE–NUTM2 fusion, which...
was previously characterised in HG-ESS as the defining genetic alteration. However, only a minority of CCSKs have this gene fusion. Of our Asian cohort of 11 CCSKs, none had the YWHAE–NUTM2 fusion. This finding is similar to that of the Japan-based study of Ueno-Yokohata et al., in which all 20 cases had BCOR ITDs and no YWHAE–NUTM2 fusion. Subsequent publications confirmed the presence of BCOR ITDs in a majority of CCSK cases, which is also the finding in our study. Gooskens et al. studied the clinical features and outcomes of CCSK with and without the YWHAE–NUTM2 fusion, and did not identify an explicit clinical phenotype for either category. Kenny et al. reported that BCOR ITD cases may have a lower incidence of high-stage disease than YWHAE–NUTM2 cases, although the differences observed did not reach statistical significance. Their report highlighted the mutual exclusiveness between YWHAE–NUTM2 fusions and BCOR ITDs in CCSKs, and identified a proportion of CCSKs (11.8%) with neither genetic abnormality corresponding to DN CCSK.

In our cohort, one of our 11 cases (9.1%) was a DN CCSK with a BCOR–CCNB3 fusion, and is the first reported case of a BCOR–CCNB3 sarcoma presenting in the paediatric kidney as a primary renal sarcoma. BCOR–CCNB3 sarcomas are currently classified in the current WHO classification of tumours of soft tissue and bone as Ewing-like primary bone sarcomas presenting in older children with primitive ovoid to spindle cell morphology that are EWSR1-fusion-negative. Unsupervised hierarchical clustering by the use of RNA-sequencing data has shown similar transcriptomic profiles between BCOR ITD-positive CCSKs and BCOR–CCNB3 fusion-positive (non-renal) sarcomas, suggesting a possible relationship between both tumour types. The up-regulated BCOR expression in all 11 CCSKs in our study supports this notion. Interestingly, the BCOR–CCNB3 case showed higher BCOR expression than BCOR ITD-positive cases. Previous studies have described the sensitivity of BCOR immunohistochemistry in BCOR ITD and BCOR translocated cases. However, this is not the finding in our study. With use of the BCOR (Abcam) and BCOR (Santa Cruz) stains, seven (64%) and three (27%), respectively, had negative BCOR immunoreactivity. Therefore, BCOR immunoreactivity cannot be used as a diagnostic immunohistochemical marker for BCOR ITD cases in the clinical diagnostic setting. CCNB3 immunoreactivity was identified in

Figure 3. Histological features and BCOR immunohistochemistry of cases 10 and 11: A, Case 10, haematoxylin and eosin-stained section. B, Case 10 with negative BCOR (Abcam) immunoreactivity. C, Case 10 with negative BCOR (Santa Cruz) immunoreactivity. D, Case 11, haematoxylin and eosin-stained section. E, Case 11 with positive BCOR (Abcam) immunoreactivity. F, Case 11 with positive BCOR (Santa Cruz) immunoreactivity.
the BCOR–CCNB3 case and not in the four other BCOR ITD CCSK cases tested, which is in keeping with previous reports.34–36,41–43 ITDs constitute a rare category of gene mutations in which a segment of a coding region of a gene is duplicated in an end-to-end manner. Although the exact mechanism remains unclear, several studies have suggested genomic instability accompanied by erroneous DNA repair mechanisms to be key elements contributing to this phenomenon.44–47 Very recently, an epidermal growth factor receptor (EGFR) ITD was reported in a DN CCSK originating from a horseshoe kidney. This had diffuse cytoplasmic EGFR immunoreactivity suggesting receptor internalisation,48 similar to FLT3 ITD in acute myelogenous leukaemia.49–51 This report thereby expands the genetic findings in DN CCSK. Since the first report of BCOR ITD in CCSK,9 BCOR ITDs have now been described in infantile soft-tissue undifferentiated round cell sarcoma (URCS),52 HG-ESS,53 primitive myxoid mesenchymal tumour of infancy (PMMTI),52 and a subset of primitive neuroectodermal tumours of the central nervous system.54

BCOR functions as a transcriptional corepressor by interacting with bel-6 via the POZ domain or by interaction with polycomb group ring finger 1 (PCGF1) through the PCGF ubiquitin-like fold discriminator (PUFD) domain as part of polycomb repressive complex 1 (PRC1).55,56 The variant PRC1 complex epigenetically silences transcription through RNF2/
RING1B-mediated monoubiquitination of histone H2AK119. Although questions regarding BCOR aberrations as driver mutations, and the mechanism of tumourigenesis in CCSK remain to be fully resolved, the consistent duplication of a 13 amino acid motif encoding the PUFD domain of BCOR and the variability of the ITD segment suggest that the ITD probably affects the binding affinity for PCGF1. To date, the only known functional in-vitro assay carried out suggests that the BCOR ITD promotes anchorage-independent growth. Also unclear at present is how CCSKs with BCOR aberrations or YWHAE–NUTM2 fusion apparently converge clinically, morphologically, and transcriptionally as studies have demonstrated a shared transcriptional signature, including elevated BCOR mRNA levels, among cases with BCOR ITDs and YWHAE–NUTM2 fusions. In addition, the round cell populations of HG-ESSs with YWHAE–NUTM2 fusions were recently reported to show BCOR immunoreactivity. The existence of both BCOR ITDs and YWHAE–NUTM2B fusions in a subset of infantile soft-tissue URCS and many PMMTIs suggests biological overlap with CCSK and the possibility that these tumours represent the soft tissue counterpart of CCSK.

In conclusion, our study of a cohort of Asian CCSK cases reveals a majority with BCOR ITDs and none with YWHAE–NUTM2 fusion, similar to another Asian series, raising the possibility of interethnic variations in the distribution of the genetic categories of CCSK between racial groups. With two BCOR antibodies (Abcam and Santa Cruz) tested, positive BCOR immunoreactivity is seen only in a proportion of CCSK cases, and BCOR immunohistochemistry therefore cannot be used as a diagnostic immunohistochemical marker for CCSK. We confirm the value of strong and diffuse nuclear cyclin D1 immunoreactivity as a sensitive diagnostic marker for CCSKs. Finally, we describe the first occurrence of a DN CCSK with the BCOR–CCNB3 fusion. DN CCSKs probably constitute a genetically heterogeneous category of cases, albeit sharing a similar histomorphology with CCSKs bearing BCOR ITDs and YWHAE–NUTM2 fusions. The full spectrum of genetic changes in DN CCSKs, their nosological status in the current diagnostic category of CCSK and their biological behaviour will remain the subjects of future studies.

Conflicts of interest
The authors declare no conflicting interests.

Author contributions
M. K. Wong performed the experiments and drafted the manuscript. C. H. Kuick and J. Sudhanshi performed the experiments. C. C. Y. Ng and V. Rajasegaran performed the NGS assays. J. Q. Lim analysed the RNA-sequencing data. E. Loh analysed the NanoString data. D. Wen Q. Lian, S. J. Aw, M. Yin, J. Ma, Z. Zhang, P. Iyer, A. H. P. Loh, S. Wang, S. G. H. Goh, T. H. Lim, A. S. T. Lim, A. H. L. Loh, P. H. Tan and K. T. E. Chang contributed and clinically, pathologically and immunohistochemically characterised the CCSK cases. K. T. E. Chang, T. Ng and A. Goytain designed the NanoString sarcoma fusion gene detection assay. B. T. Teh and K. T. E. Chang conceived and oversaw all aspects of the study.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Table S1. (A) Primers for BCOR ITD. (B) Primers for BCOR qPCR and gene fusion validation.

Table S2. List of the four pools of primers covering the entire BCOR gene (NGS).

Figure S1. Chromatograms from the Sanger sequencing validation of the identified fusion genes in case 2. Red lines indicate the translocation breakpoints.

Data S1. Supplementary protocols.

Data S2. Histological images and BCOR immunohistochemistry.